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MINIREVIEW

# Cytochrome *c*, a hub linking energy, redox, stress and signaling pathways in mitochondria and other cell compartments

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Cytochrome *c* (CYTc) is a soluble redox-active heme protein that transfers electrons from complex III to complex IV in the cyanide-sensitive mitochondrial respiratory pathway. CYTc biogenesis is a complex process that requires multiple steps until the mature active protein is obtained. CYTc levels and activity are finely regulated, revealing the importance of this protein not only as electron carrier but also in many other processes. In this article, we describe the role of CYTc in mitochondrial respiration, from its canonical role as electron carrier for ATP production to its involvement in protein import and the stabilization of respiratory complexes and supercomplexes. In plants, CYTc is connected to the synthesis of the antioxidant ascorbate and the detoxification of toxic compounds. Finally, CYTc is also a multi-functional signaling molecule that influences the balance between life and death, acting in energy provision for cellular functions or triggering programmed cell death. The confluence of several metabolic routes into a single protein that links redox reactions with energy producing pathways seems logical from the point of view of cellular economy, control and organization.

## Introduction

Mitochondria are semi-autonomous organelles that possess their own genome and are able to synthesize a small number of proteins essential for respiratory functions. Mitochondria are the main fuel stations of eukaryotic cells as they produce ATP through *oxi*dative *phos*phorylation (OXPHOS). In addition, they are vital for cellular homeostasis, participating in diverse metabolic pathways, calcium signaling and reactive oxygen species (ROS) buffering or production. These functions link mitochondrial activity with the environment and with other cell compartments like the endoplasmic reticulum, the nucleus and, in photosynthetic organisms, the chloroplast (Welchen et al. 2014). Defects in mitochondrial function are responsible for serious pathologies in humans and deleterious alterations in plants (Kühn et al. 2015, Mansilla et al. 2015, Soto et al. 2015, García et al. 2016). Mitochondria also behave as signaling organelles. The release of ROS and cytochrome *c* (CYTc), among other molecules, is used by mitochondria to send retrograde signals that impact at the whole cellular level (Martínez-Fábregas et al. 2014, Cagin et al. 2015, Van Aken and Van Breusegem 2015).

OXPHOS is fulfilled by the mitochondrial electron transport chain (mETC), strategically positioned in the

*Abbreviations* – ASA, ascorbic acid; CCHL, CYTc heme lyase; CCM, cytochrome C maturation; CcO, cytochrome *c* oxidase; CL, cardiolipin; CYTc, cytochrome *c*; DLDH, D-lactate dehydrogenases; GLDH, L-galactono-1,4-lactone dehydrogenase; HCCS, holo-CYTc synthase; IMS, intermembrane space; mETC, mitochondrial electron transport chain; MG, methylglyoxal; OXPHOS, *oxi*dative *phos*phorylation; PCD, programmed cell death; ROS, reactive oxygen species.

mitochondrial inner membrane, connecting the matrix and the intermembrane space (IMS), and which is coupled to ATP synthesis. The mETC is composed of four multisubunit protein complexes (I to IV). In addition, CYT*c*, a small heme protein located in the IMS, participates as electron carrier between complexes III and IV. Plants also have alternative respiratory routes, represented by NAD(P)H-dehydrogenases and the alternative oxidase (AOX), which are responsible for preventing the accumulation of reducing power in the mitochondrial matrix or the overreduction of mETC components under stress conditions (Van Aken et al. 2009).

In this article, we focus our attention on the sole protein component of the respiratory pathway that acts as a single small molecule: CYT*c*. We describe the steps that regulate its biogenesis and its role in mitochondrial respiration, from its canonical role as electron carrier for ATP production to its involvement in protein import and the stabilization of respiratory complexes and supercomplexes. CYT*c* is also a multi-functional signaling molecule, acting as a linker between mitochondrial activity and the cellular environment. CYT*c* triggers programmed cell death (PCD) and, in plants, it is connected to the synthesis of the antioxidant ascorbate and the detoxification of toxic compounds. A role of CYT*c* as a control point of multiple redox processes seems logic from the standpoint of cellular fitness, as regulation of this single protein may be used by the cell to modulate whole respiratory activity. Fig. 1 summarizes all the aspects related to the CYT*c* biology and functions that were considered and will be described throughout this revision.

## Mechanisms of CYT*c* biogenesis and regulation

### CYT*c* biogenesis: regulation of CYTC gene expression

The expression of genes encoding CYT*c* was studied mainly in Arabidopsis, which contains two *CYTC* genes that are differentially expressed and regulated. Both *CYTC* genes are induced by metabolizable sugars, nitrogen sources and light (Welchen et al. 2002). The *CYTC-1* gene is expressed in tissues of high cell division rate and seems to be the isoform responsible for basal protein levels, while *CYTC-2* is transcriptionally upregulated during stress conditions (Welchen and Gonzalez 2005, Welchen et al. 2009). Expression of *CYTC-1* is governed by promoter elements named site II (TGGGCC/T) and a telo-box (AAACCCTA) (Welchen and Gonzalez 2005, 2006). Site II elements coordinate the expression of genes encoding components of the different complexes of the mETC and regulate the global induction of OXPHOS genes mainly by carbohydrates, thus connecting gene expression with cellular demands (Gonzalez et al. 2007). By an evolutionary process known as neofunctionalization, *CYTC-2* incorporated the specific regulatory elements G-box and ACGT, leading to specialized expression patterns and conferring to the *CYTC-2* gene the ability to respond to several environmental and metabolic factors (Welchen et al. 2009). Thus, while *CYTC-1* is usually expressed in tissues of highly energy demand, *CYTC-2* expression is widespread throughout the vascular plant tissues and exert their main function in response to several stress situations.

### CYT*c* biogenesis: synthesis and maturation of CYT*c*

Mitochondria possess two *c*-type cytochromes, the soluble redox-active heme protein CYT*c* that transfers electrons between complex III (cytochrome *c* reductase) and complex IV (cytochrome *c* oxidase, C*c*O) and the inner membrane anchored $CYTc_1$ that constitutes an integral part of complex III. Structurally, both contain covalently bound heme, which is attached by two thioether bonds established between two reduced cysteines present in the conserved apocytochrome *c* (apo-CYT*c*) heme-binding motif ($C_1XXC_2H$), and two vinyl groups present in heme *b*. Covalent ligation of the heme cofactor confers stability to the apo-CYT*c*, avoiding its rapid degradation.

Biogenesis of *c*-type cytochromes is an intricate process. First, apo-CYT*c* and heme should be translocated from their site of synthesis (cytosol and the mitochondrial matrix or the chloroplast, respectively) to the assembly site in the IMS. Once in the IMS, apo-CYT*c* and heme must be maintained in a reduced redox state until the heme attachment reaction occurs. There are five CYT*c* maturation pathways that have been described in different organisms. System I, also referred as CCM (cytochrome C maturation) pathway, was first described in $\alpha$- and $\gamma$-proteobacteria, and is found in bacteria, archea and plant mitochondria (Giegé et al. 2008). System II (Ccs, CYT*c* synthesis) is used by $\beta$-, $\delta$- and $\varepsilon$-proteobacteria, Gram-positive bacteria, algae and is also is present in chloroplasts. System III is characteristic of yeast and mammalian mitochondria where enzymes named CYT*c* heme lyase (CCHL) or holo-CYT*c* synthase (HCCS) participate in apo-CYT*c* transport and heme attachment. Systems I to III were described on the positive (-*p*) side of bioenergetics membranes of bacterial periplasm, chloroplast lumen and mitochondrial IMS. Finally, two other systems (IV and V) were recently described for the biogenesis of unusual *c*-type cytochromes. System IV catalyzes heme attachment through a single thioether bond on the negative (-*n*) side of bioenergetic membranes presents in bacterial

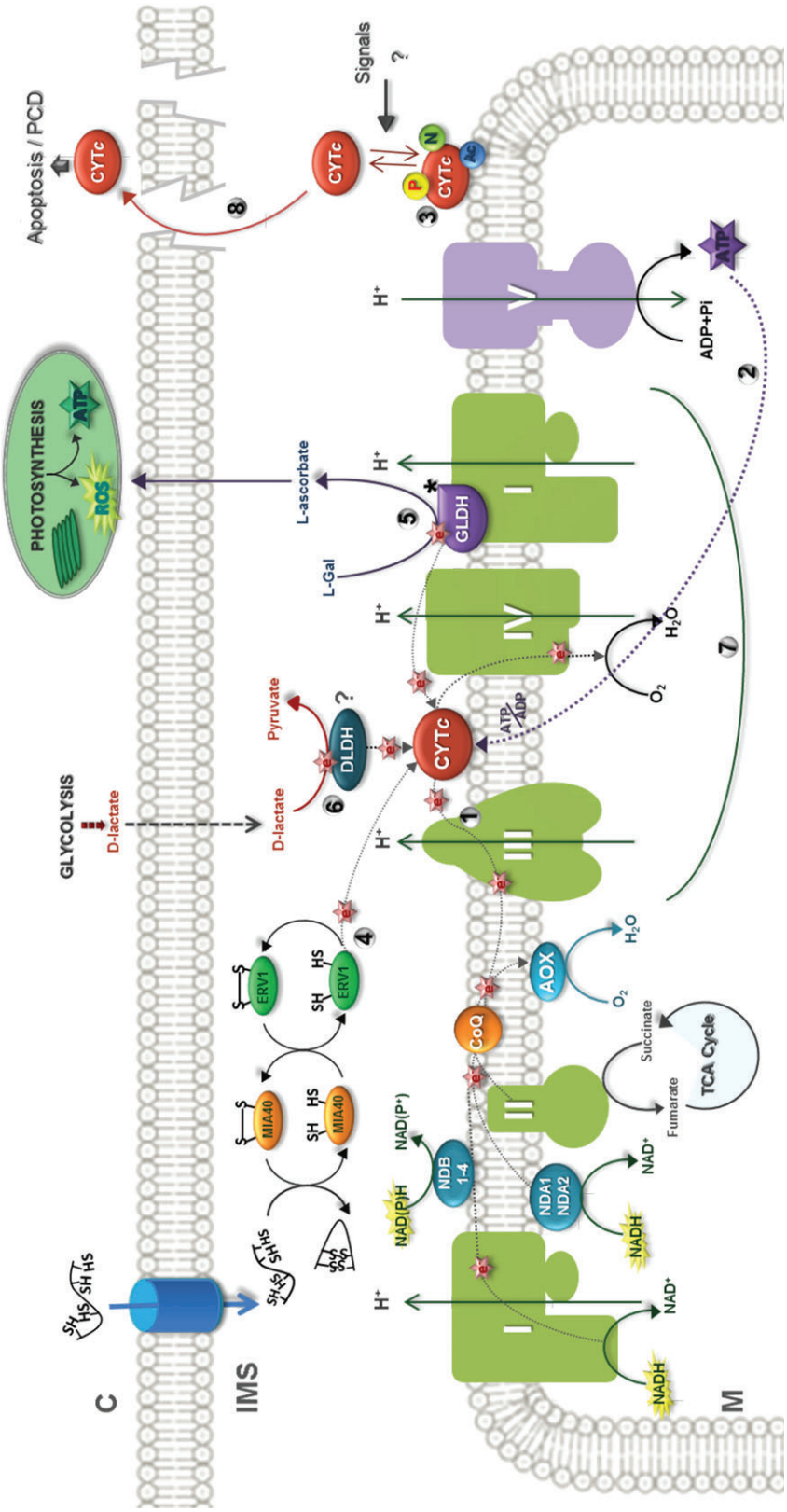


**Fig. 1.** Legend on next page.

cytoplasm and plastid stroma. This system is found in all organisms with oxygenic photosynthesis but not in Firmicutes (e.g. *Bacillus subtilis*). System V is described in mitochondria of Euglenozoans, were the heme is hexacoordinated with a single tioether bond (Verissimo and Daldal 2014).

There are marked differences in the last step of the CYT*c* maturation pathways described in yeast, mammals and plants. While yeast and mammals evolved a simple mechanism based on the enzymes CCHL or HCCS (Babbitt et al. 2015), the CCM system involves up to nine membrane and periplasmic assembly proteins (CcmABCDEFGHI) working together to achieve heme ligation and complete CYT*c* biogenesis (Verissimo and Daldal 2014). Plant mitochondria have conserved the inherited type-I maturation pathway from their prokaryotic ancestor with some remarkable differences. Bacterial homologues to CcmD, CcmI, DsbD/CcdA and CcmG proteins are absent and CcmF is split in multiple genes encoding different domains (AtCcmFN1, AtCcmFN2 and AtCcmFC in Arabidopsis) (Giegé et al. 2008). In Arabidopsis, heme delivery is exerted by the CcmABCE complex, being AtCCMA, AtCCMB and AtCCMC transmembrane proteins and AtCCME a heme chaperone that ensures a stable heme reservoir in the IMS. AtCCMH is an essential redox component of the maturation pathway, which interacts with apo-CYT*c* to maintain its cysteines in a reduced state. Together with AtCcmFN1, AtCcmFN2 and AtCcmFC, AtCCMH integrates a high molecular heme lyase activity complex involved in the last step of CYT*c* maturation (Meyer et al. 2005). It can be observed that CYT*c* biogenesis is a complex process in plants and that multiple steps are required until the mature active protein is obtained. Regulation of CYT*c* biogenesis may be used by the cell to regulate CYT*c* levels according to physiological parameters. Until now, however, it is not known if this level of regulation operates in plant cells.

## Regulation of CYT*c* activity: ATP

It was shown that CYT*c* contains a high affinity ATP binding site. It has been proposed that ATP may regulate the rate of electron transport, acting as a feedback inhibitor of the reaction between CYT*c* and C*c*O, due to structural and/or electrostatic changes of both proteins. The key amino acid involved in this regulation in mammals is Arg91 (Tuominen et al. 2001), also present in CYT*c* proteins from plants (Fig. 2). This regulatory pathway may help to adjust the activity of the respiratory chain to the energetic state of the cell.

## Regulation of CYT*c* activity: posttranslational modifications

Many different posttranslational modifications have been described for non-plant CYT*c*. Several of them are listed below.

### *Phosphorylation*

Mass spectrometry and phosphomimetic experiments demonstrated the relevance of phosphorylation of residues Tyr48 and Tyr97 in CYT*c* from cow liver and heart (Lee et al. 2006, Yu et al. 2008). Tyr48 and Tyr97 are exposed to the protein surface, near the heme binding site. Phosphorylation of both residues produces structural changes that regulate respiration and apoptosis. Thus, under healthy conditions, phosphorylation may originate a decrease in mitochondrial OXPHOS activity to avoid excess ROS production (Lee et al. 2006, Yu et al. 2008). Moreover, Lys7 is essential for the interaction between CYT*c* and the human protein Apaf-1 during apoptosome formation. Lys7 is located next to Tyr97 and it was recently demonstrated that phosphorylation of Tyr97 prevents the release of CYT*c* from mitochondria in vivo (Sanderson et al. 2013). In addition, Tyr48 phosphorylation might affect the cardiolipin (CL) peroxidase activity of CYT*c*, thus preventing induction of apoptosis (Yu et al. 2008). Under stress conditions, both sites (Tyr48 and Tyr97) would be dephosphorylated to increase respiration, ROS production and CL oxidation with the consequent detachment of CYT*c* from the mitochondrial membrane, caspase activation and apoptosome formation (Pecina et al. 2010). Within the first events that trigger apoptosis, CYT*c* is activated by presence of $H_2O_2$, CL

**Fig. 1.** CYT*c* is a multifunctional protein. (1) CYT*c* is a soluble heme protein of the inner membrane space that transports electrons between complexes III and IV. (2) Its activity can be regulated by ATP, acting as a feedback inhibitor of the reaction between CYT*c* and complex IV or cytochrome *c* oxidase. (3) Many different posttranslational modifications [phosphorylation (P), nitration (N) and acetylation (Ac)] have been described for CYT*c*. (4) CYT*c* participates as electron acceptor in the alternative mitochondrial import pathway represented by MIA40 and ERV1 proteins, (5) in the final step of the synthesis of the antioxidant compound ascorbic acid, and (6) in the oxidation of D-lactate to pyruvate during the detoxification of methylglyoxal produced during glycolysis. (7) CYT*c* is also involved in the assembly and/or stabilization of respiratory complexes and supercomplexes in yeast, mammals and plants. (8) CYT*c* has the final decision between life and death as it can exit from mitochondria and play an active role in triggering programed cell death. CoQ, coenzyme Q/ubiquinone; C, cytosol; M, matrix; NDA1/NDA2, internal alternative NADH dehydrogenases; NDB1/NDB2, external alternative NADH dehydrogenases.

**Fig. 2.** Legend on next page.

redistribution in the mitochondrial membrane and the consequent alteration of the electrostatic CL/CYTc interactions, to induce the CL-specific oxygenase activity of CYTc. The 'switch on' of this CL-peroxidase role precedes the loss of tertiary structure of the heme-protein and generates CL-hydroperoxides, absolutely necessary for release of pro-apoptotic factors (Kagan et al. 2005, Belikova et al. 2006). While Lys7 is not conserved in plants, both tyrosine residues are highly conserved among eukaryotes (Fig. 2), so their potential role as target sites for regulation by cell signaling pathways should be explored.

Thr28 and Ser47 are two other phosphorylation sites that have been reported in CYTc from human skeletal muscle (Zhao et al. 2010). While Ser47 is highly conserved, position 28 is mainly occupied by Gln in plants (Fig. 2). Recently, by using phosphomimicking CYTc mutants in residues 28 and 47, a role of phosphorylation in both residues in the affinity of CYTc for CL and in the consequent CYTc CL-peroxidase activity was proposed. Moreover, residue 47 was pointed as relevant for caspase activation during apoptosis signaling pathway (Guerra-Castellano et al. 2016).

### *Acetylation*

In mammals, CYTc is among the 20% of mitochondrial proteins modified by acetylation. This modification may cause an alteration in protein conformation, affecting its redox function (Zaidi et al. 2014). Particularly in Arabidopsis, CYTc was the unique component of the mETC found to be modified by acetylation, specifically in Lys72 (Finkemeier et al. 2011). Lys72 is essential for CYTc anchoring to the membrane surface and for binding to its redox partners (complex III and CcO) in mammals (Zaidi et al. 2014). Although not experimentally demonstrated, Lys acetylation could be important for the regulation of CYTc activity in plants.

### *Nitration*

Another important modification observed in CYTc is nitration. In general, nitration prevents the progression of the apoptosis signaling pathway. Specific nitration of Tyr residues 46 and 48 induces the specific degradation of cytochrome and avoids formation of a functional apoptosome (Díaz-Moreno et al. 2011, García-Heredia et al. 2012). Moreover, nitration of solvent-exposed Tyr74 enhances CYTc peroxidase activity and blocks the ability to activate caspase-9 for progression of apoptosis (García-Heredia et al. 2010). All residues are present in the plant proteins but the presence or regulatory significance of this modification in vivo has not been explored.

## CYTc is a multifunctional protein

### Canonical function: respiration and energy production

CYTc is a soluble heme protein of the IMS that transports electrons between cytochrome $bc_1$ reductase (complex III) and CcO (complex IV). Under physiological conditions, this reaction constitutes the rate-limiting step of the mETC in mammalian cells (Acín-Pérez 2008, Hüttemann et al. 2011). CYTc is essential for aerobic energy production and both knockout of *CYTc* genes or defects in the maturation of the holoprotein are lethal in plants due to defects in embryogenesis (Meyer et al. 2005, Welchen et al. 2012). CYTc knockout mice die around midgestation (Li et al. 2000) and alterations in CYTc levels or in signaling pathways including this protein were connected to different severe human diseases (Hüttemann et al. 2011). Phenotypically, *CYTc* knockdown plants exhibit smaller size, slower growth rate and a delay in the onset of symptoms related to natural senescence (Welchen et al. 2012). These defects may be caused by decreased energy production and indicate that the function of the CYTc-dependent respiratory pathway is required for normal plant growth.

As we mentioned before, the expression of *AtCYTc*-1 gene is controlled at promoter level by site II elements, which are also present in genes encoding ribosomal proteins and chloroplast proteins and may be related to the expression of genes required for cell proliferation and

**Fig. 2.** Sequence alignment of CYTc proteins from plants and mammals. CYTc sequence alignment (A) and WEBlogo (B) showing conservation in several relevant residues in plants and mammals. CYTc sequences from plants are boxed in green. The conserved cysteine residues involved in covalent heme binding are highlighted in red. The blue asterisk indicates Lys7, required for the interaction between CYTc and the human protein Apaf-1 during apoptosome formation. This residue is not conserved in CYTc from plants. Phosphorylated residues in mammalian CYTc (Thr28, Ser47, Tyr48 and Tyr97) are pointed out with orange arrows. Position 28 is mainly occupied by Gln in plants. The blue arrow marks Lys72, susceptible to acetylation and involved in the anchorage of the protein to the inner membrane. Green arrows show residues modified by nitration (Tyr46, Tyr48 and Tyr74). The purple arrow points to Arg91, involved in the feedback inhibition of mitochondrial electron transport by ATP in mammals. Plant CYTc sequences were collected from the Phytozome database (http://phytozome.jgi.doe.gov/pz/portal.html). Sequences from human (NP_061820), mouse somatic (s) and testes-specific (t) isoforms (P62897 and NP_034191, respectively) were downloaded from the NCBI database (http://www.ncbi.nlm.nih.gov/). Protein sequences were aligned using ClustalW2.0 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The WEBlogo was constructed using the site http://weblogo.berkeley.edu/logo.cgi based on the sequence alignment shown in (A).

growth. A survey in the TAIR10 loci upstream (−500 bp) DNA sequence database using the PATMATCH web tool (https://www.arabidopsis.org/cgi-bin/patmatch/nph-patmatch.pl) allowed us to identified 5216 Arabidopsis genes containing at least two site II in this limited fragment of their promoter region. Gene Ontology (GO) term enrichment analysis using the Virtual Plant database (http://virtualplant.bio.nyu.edu/cgi-bin/vpweb/virtualplant.cgi, Katari et al. 2010) confirmed that site II elements are enriched in genes encoding proteins involved in translation, RNA metabolism, protein transport and the biogenesis of cellular components (Table S1). Then, a link between function of CYT*c* in energy production and cell growth was reinforced on the basis of the enrichment of this regulatory promoter element.

## Additional CYT*c* functions

### Protein import

Mitochondria contain a double membrane separating three chemically different soluble compartments, the cytosol, the IMS and the matrix. Mitochondrial proteins encoded by the nuclear genome must be imported into the specific organelle compartment to fulfil their activity. The mitochondrial import apparatus is classically composed of translocase of the outer membrane (TOM) and translocase of the inner membrane (TIM) complexes and a set of components specific from plants. An alternative import mechanism is represented by the mitochondrial import and assembly pathway (MIA), consisting of proteins MIA40 and ERV1 that are located in the IMS. This pathway is used for the import of IMS proteins that contain cysteines arranged in $CX_9C$ or $CX_3C$ motifs. Through a disulphide relay system, cysteine residues of the imported proteins are oxidized and the conformational changes due to the formation of disulphide bonds constitute the driving force for moving proteins into mitochondria (Riemer et al. 2011). The cysteine residues are recognized by redox-active cysteines presents in the oxidoreductase MIA40, which are maintained in an oxidized state by the sulfhydryl oxidase ERV1. In the last step, ERV1 is re-oxidized by interaction with CYT*c*, which drives electrons to the mETC at the level of C*c*O (Bihlmaier et al. 2007).

The MIA40-ERV1-CYT*c* pathway is an evolutionary conserved import mechanism. There are more than 13 proteins with $CX_9C$ motifs in yeast, mammals and plants (Longen et al. 2009). The best-characterized members of this group are COX17 and COX19, two copper binding proteins involved in the delivery of this metal for C*c*O biogenesis (García et al 2014, 2016). Potential homologues of ERV1 and MIA40 were described in Arabidopsis (Carrie et al. 2010). However, MIA40 does not seem to be an essential protein in plants, possibly indicating the existence of alternative routes for import of $CX_9C$ proteins.

While the mechanism of electron transfer from ERV1 to CYT*c* remains unclear, experimental observations demonstrated that oxidized CYT*c* accelerates the oxidation of MIA40 in vitro and in vivo. Furthermore, mutations in C*c*O or inhibition of the mETC by cyanide prevent MIA40 oxidation (Bihlmaier et al. 2007). It has also been shown that import kinetics depends on the redox state of the glutathione pool and the electrochemical potential across the inner membrane. In this way, the mETC, through CYT*c* action, acts both as a sink of electrons and as a regulatory step for protein import through the MIA pathway.

### Stability of respiratory complexes and supercomplexes

Using blue-native polyacrylamide gel electrophoresis (BN-PAGE) defined supercomplexes that have a $I + III_2$, $III_2 + IV_{1-2}$, $I + III_2 + IV_{1-4}$ and $V_2$ composition could be described in the mitochondrial inner membrane (Eubel et al. 2004, Dudkina et al. 2006). It has been shown that these superstructures are related to the stability of individual complexes and promote a more efficient electron transfer (Lapuente-Brun et al. 2013). In mouse mitochondria, respiratory complexes are arranged in supercomplexes called respirasomes. These include the presence of coenzyme Q (CoQ) and CYT*c* into their constitution, so they can continue their respiratory activity even when are isolated from the inner membrane (Acín-Pérez et al. 2008).

Even though CYT*c* is a soluble protein, interactions with both polar groups of phospholipids and strong hydrophobic interactions with the inner membrane were demonstrated (Benard et al. 2008, Althoff et al. 2011). Recently, Moreno-Beltrán et al. (2014) demonstrated two binding site of plant CYT*c* to the head soluble domain of $CYTc_1$, present in complex III. This extra binding site of CYT*c* to the complex III facilitates the electron transfer to complex IV. These findings support a substrate channeling mechanism in which a pool of CYT*c* binds more strongly to the inner membrane, building a bridge of electrons in close contact with complexes III and IV instead of carrying electrons by random diffusion across the IMS (Acín-Pérez et al. 2008, Vempati et al. 2009). In plants, supercomplexes conformed by complexes I, III and IV, adopting the stoichiometry $I:III_2:IV$ in a configuration that may allow CYT*c* binding, were described (Eubel et al. 2004, Dudkina et al. 2006, Genova and Lenaz 2014).

Binding of CYTc to supercomplexes may explain the fact that CYTc deficiency causes a decrease in the amount of complex IV in yeast, mammals and plants. In the case of mouse fibroblasts, CYTc is also required for complex I assembly or stability (Vempati et al. 2009). In yeast, it has been suggested that CYTc is mainly required for complex IV assembly and that it also affects CcO stability (Barrientos et al. 2003). In plants, CYTc deficiency causes a decrease in the levels and activity of complex IV, without affecting the amount or composition of the other complexes (Welchen et al. 2012).

### Ascorbic acid synthesis

In addition to the benefits conferred to eukaryotic cells by aerobic respiration, this also brought the disadvantage of ROS generation. To protect themselves from the harmful effects of these toxic compounds, cells evolved systems composed by multiple defense barriers, among which molecules with antioxidant activity are particularly important. Ascorbic acid (ASA) plays a critical role in protecting cells against damaging ROS. ASA is synthesized through different routes, being the last step catalyzed by the terminal enzyme GULO (L-gulonolactone oxidase) in animals and by the alternative mitochondrial enzyme GLDH (L-galactono-1,4-lactone dehydrogenase) in plants (Wheeler et al. 2015). ASA synthesis is connected in plants to the mETC at two different levels. First, GLDH is a mitochondrial enzyme detected in assembly intermediates of complex I and is absolutely required for the assembly and accumulation of this respiratory complex (Schertl et al. 2012, Schimmeyer et al. 2015). Second, GLDH uses CYTc as electron acceptor during the ASA synthesis reaction (Bartoli et al. 2000, Millar et al. 2003). In this sense, it was observed that GLDH activity was decreased to 60% of wild-type levels in Arabidopsis CYTc knockdown mutants (Welchen et al. 2012). Bearing in mind the multiple roles of ASA and that a significant proportion of ROS is produced during photosynthesis in the chloroplast, the integration of L-galactono-1,4-lactone oxidation and mETC activity via CYTc could serve to coordinate ASA synthesis and metabolism with the cellular energy and redox state.

### D-lactate oxidation

D-lactate is produced by the glyoxalase system that catalyzes the metabolization of the cytotoxic compound methylglyoxal (MG), primarily formed as a by-product of glycolysis during different (salt, drought, cold and heavy metal) stress conditions. D-lactate is rapidly metabolized to pyruvate in eukaryotic cells through the action of enzymes known as D-lactate dehydrogenases (DLDH). Engqvist et al. (2009) demonstrated in vitro that the enzyme DLDH from Arabidopsis catalyzes the oxidation of D-lactate to pyruvate using CYTc as electron acceptor. Although this must be corroborated in planta, it is conceivable that the oxidation of D-lactate could be connected to the mETC through the electron carrier CYTc. Derivation of electrons from MG detoxification could also be a signal for regulating mitochondrial respiratory activity in order to overcome the unfavorable growth condition generated by stress.

### Life and death converge on CYT*c*: PCD

In addition to providing energy for cellular functions and growth, another, somewhat contradictory function has been demonstrated for CYTc: to promote cell death when necessary during normal development or after conditions of irreparable damage imposed to the cell.

PCD is a suicide mechanism occurring during normal development in multicellular organisms (from embryogenesis to death) and also constitutes a defensive response against pathogen attack (Van Hautegem et al. 2015). In plants, mitochondrial ROS are essential for the progression of PCD and the chronology of events shares similarities with those described in mammals. Perception of death signals or different stimuli (i.e. UV light, $H_2O_2$, several stresses, pathogen attack and developmental cues) causes massive mitochondrial ROS production, activation of MAPK cascade loss of mitochondrial transmembrane potential (MTP), mitochondrial swelling and, finally, activation of caspase-like activities and PCD (Lord et al. 2013, Rantong and Gunawardena 2015). Unlike what happens in mammals, plants possess metacaspases that cleave their substrates after Arg and Lys residues rather than Asp and are regulated by $Ca^{2+}$. Also vacuolar processing enzymes (VPEs) with caspase-like Asp-specific protease activities, phytases and saspases are active during PCD (Van Aken and Van Breusegem 2015). Except phytases, that are recruited active, all others are produced as inactive proteins that are autocatalytically processed into active forms induced by suitable conditions like optimal pH and a high $Ca^{2+}$ concentration (Rantong and Gunawardena 2015)

In mammals, it was absolutely established that CYTc is essential for the outcome of certain types of PCD (Ow et al. 2008, Rantong and Gunawardena 2015). In plants, the release of both mitochondrial CYTc and chloroplastic cytochrome *f* (CYT*f*, another *c*-type cytochrome) was connected to PCD execution (Kim et al. 2012, Wang et al. 2014). There is evidence about the presence of CYTc in the cytosolic compartment, obtained by subcellular protein fractionation followed by western blot analysis. This was demonstrated in maize cells

treated with mannose or infected with *Agrobacterium* (Stein and Hansen 1999, Hansen 2000), in wheat roots under anoxic stress (Virolainen et al. 2002), in tobacco Bright-yellow-2 cells after heat shock treatment (Vacca et al. 2004) and in Arabidopsis after PCD induced by aluminum phytotoxicity (Li and Xing 2010), among other examples listed in Diamond and McCabe (2011). Recently, the existence of a common signalosome for PCD in humans and plants was proposed based on the interaction of CYT*c* with a wide range of targets of PCD by proteomic analysis (Martínez-Fábregas et al. 2013, 2014). Nonetheless, despite the demonstrated release of CYT*c* in plants, in vivo evidence of the presence of this heme protein outside mitochondria playing an active role in PCD execution is lacking. Unlike mammals, plants also have chloroplasts that produce ROS and may have a role in triggering PCD (Kim et al. 2012). In this sense, Van Aken and Van Breusegem (2015) proposed models in which mitochondria and chloroplasts efficiently cooperate in the execution of plant PCD.

## Conclusion

The many roles of CYT*c* discussed in this article are schematized in Fig. 1. CYT*c* is a soluble heme protein of the IMS that transports electrons between complexes III and IV (Fig. 1). CYT*c* is essential for aerobic energy production and both defects in the maturation of the holoprotein or knockout of *CYTC* genes are lethal due to defects in embryogenesis (Li et al. 2000, Meyer et al. 2005, Welchen et al. 2012). CYT*c* deficiency is also connected to several human diseases (Hüttemann et al. 2011). CYT*c* activity is regulated by ATP, the final product of OXPHOS, acting as a feedback inhibitor of the reaction between CYT*c* and C*c*O (Fig.1). Many different posttranslational modifications have also been described for non-plant CYT*c* (Fig. 1). Among them, phosphorylation, nitration and acetylation (also demonstrated in plants) are involved in regulating CYT*c* activity and its role in energy production and PCD. CYT*c* participates as electron acceptor in the alternative mitochondrial import pathway represented by MIA40 and ERV1 (Fig. 1), in the final step of the synthesis of the antioxidant compound ASA (Fig. 1), and in the oxidation of D-lactate to pyruvate during the detoxification of MG (Fig. 1). CYT*c* is also involved in the assembly and/or stabilization of respiratory complexes and supercomplexes in yeast, mammals and plants (Fig. 1). At the end, CYT*c* has the final decision between life and death as, according to several parameters, it can exit from mitochondria and play an active role in triggering PCD (Fig. 1). Rather than separate, independent processes, it is likely that the many pathways that use CYT*c* are interconnected, thus converting this protein in a hub that may be used by the cell to integrate energy, redox and stress-related parameters into growth responses.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Biological process and subcellular localization GO term enrichment of Arabidopsis genes containing at least two site II elements in their promoter region.

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